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**13. ABSTRACT** *(Maximum 200 Words)*

This project aims to develop catalytic antibodies that will allow efficient DNA transfer by promoting DNA site-specific recombination. Such antibodies may provide a very powerful means to manipulate DNA transfer at defined sequences with obvious implications for gene therapy of breast cancer. In order to engineer enzymes that catalyze the recombination, we immunized mice with a synthetic DNA Holliday structure and screened over 5,000 hybridomas. We had candidate clones that seemed to either resolve or bind to the structure in primary screenings. Unfortunately we have not been able to isolate clones that possess the properties after expansion and purification. For constructing fab expression library we encountered difficulties in amplifying Fab cDNA from immunized mouse splenic mRNA. We have made a few primary libraries. We are in the beginning of screening the libraries for the Fabs that resolve the structure. This is a very technical and labor challenging project. I feel that our resources are limited to successfully complete the project. Successful completion of this project will provide a new technology of DNA transfer for both biomedical research and therapeutic purposes.

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Barbara Thompson 22Nov 99

PI - Signature Date

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## INTRODUCTION

Gene therapy is an emerging chemotherapeutic approach to cancers, and breast cancer is a particularly attractive target in this regard (reviewed by Avalosse *et al.*, 1995, Mastrangelo *et al.*, 1996). Most gene transfer techniques result in low transfection efficiency and random insertion of the transgene into host genome. High efficiency gene transfer into defined genomic sites is critical for therapeutic purposes (see review by Yanez and Porter, 1998). Ideal genetic therapy would involve the transfer of a functioning gene into a defined site on the genome in target cells. DNA site-specific recombination would allow insertion of a functional gene into a specified genomic locus, thereby placing the transgene under normal control. Antibodies have inherent structural specificity, which is advantageous for generation of nucleotide sequence-specific reagents. We propose to generate sequence specific antibodies that possess integrase activity and are able to catalyze recombination at defined nucleotide sequences. This pilot project aims to demonstrate the feasibility of creating such catalytic antibodies during the period from September, 1997 to September, 2000.

## BODY

We have determined that the Holliday structure (Figure 1*) is antigenic by using a synthetic DNA Holliday structure to elicit immune response in mice. DNA Holliday structure is the intermediate of integrase-mediated site-specific recombination. Formation of the Holliday structure and its subsequent resolution into two strand-exchanged products is mediated by an integrase, which is a single polypeptide enzyme with no known cofactors. All integrases contain a highly conserved amino acid tetrad, Arg-His-Arg-Tyr (Abremski and Hoess, 1992). A typical integrase is the 50kDa FLP protein, which catalyzes site-specific recombination of 2μm plasmid in Saccharomyces cerevisiae (Gates and Cox, 1988). A single protein mediates the resolution and formation of the Holliday structure. It is reasonable that an antibody that resolves the Holliday structure will also catalyze its formation, since the chemistry of formation is identical to that of resolution. Antibodies that catalyze the formation and resolution of the Holliday structure will thus be engineered integrases. These integrases will be able to catalyze site-specific recombination and provide very important new reagents for DNA transfer. The target sequence specificity will be derived from the antigen used to elicit the immune response.

We have designed a set of 4 oligonucleotides (Oligo1, 60 nucleotides (nt), Oligo2, 42nt, Oligo3, 53nt, and Oligo4, 71nt) (Figure 2). A Holliday structure (hereinafter called HS) can be annealed from these four oligonucleotides as shown in Figure 3. HS has four arms with different lengths (Figure 3). Five hybrids (HS, R, S, Y, and Z) are predicted from annealing of the 4 oligonucleotides (Figure 3). However, we routinely detected HS, Y and Z in the mixture of these oligonucleotides as shown in Figure 4. The resolution of HS will generate two alternative pairs of double-stranded DNAs (Figure 5), which is the basis of our integrase assay. HS can elicit immune response in mice as shown in Figure 6. The anti-serum contained antibodies that specifically bind to the cruciform center structure of HS as shown in Figure 7.

Our short-term goal is to test the hypothesis that catalytic antibodies can recombine DNA fragments in vitro, and to develop catalytic antibodies that can do so. Three technical objectives were proposed: **1**) Produce hybridomas that secrete antibodies against the Holliday structure and screen for monoclonal antibodies that resolve the structure; **2**) Construct an Fab expression library and screen for Fabs that resolve the Holliday structure; **3**) Determine if these catalytic antibodies (Fabs) recombine DNAs of defined sequences in vitro. We are now pursuing the first two technical objectives as planed. We have not obtained a positive clone so far. As soon as we obtained a positive clone we will test it as stated in technical objective **3**.

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* All figures are attached to Appendices.

The following are the Technical Objectives originally proposed in association with each task approved in the Statement of Work with an additional section ***Results and Discussion*** describing what has been accomplished and the reason for changing the original plan.

Technical Objective **1**: Production of Hybridomas and Screening of Monoclonal Antibodies That Resolve the Holliday Structure:

Serum from HS-immunized mice contains a mixed population of antibodies. Our initial goal is the preparation of specific monoclonal antibodies with the desired specificity. We will isolate and screen hybridomas to this end. We have designed a functional assay to screen for antibodies that resolve HS, as illustrated in Figure 5. The decision not to use ELISA is based on the use of HS both as the antigen and as the substrate for the screening assay. An antibody with enzymatic activity will rapidly dissociate from its product after resolution of HS. ELISA detects stable antibody-antigen complexes. Since we expect the antibody-product complex to be unstable, we should not detect a catalytic antibody by ELISA. The catalytic activities of HS-specific antibodies will be determined by a two-step screening method as described below. The substrate used for the first step of screening is HS labeled with $^{32}P$ on the 5'-ends of the three shorter arms. Biotin will be affixed to the 5'-end of the longest arm, to allow flexibility to the substrate after it is bound to a solid surface. The biotin will be used as an anchor to immobilize the substrate onto a microtest plate covered with streptavidin. Supernatants from individual hybridomas will be incubated with the immobilized substrate. After a period of incubation, part of the reaction mixture from the individual supernatants will be counted with a liquid scintillation counter to detect the release of $^{32}P$ from the bound substrate. The release of $^{32}P$ indicates that the culture supernatant contains one of the following activities: 1) phosphatase; 2) exonuclease; 3) endonuclease; or 4) integrase. The reaction mixtures that contain released $^{32}P$ will be adjusted to 10% (v/v) of cold trichloroacetic acid (TCA) to precipitate macromolecule-bound $^{32}P$ (i.e. labeled DNA fragments). TCA does not precipitate ortho-phosphate or nucleotide monophosphates that would be released by phosphatases or exonucleases. The TCA-precipitated DNA will be filtered onto Whatman GF/C glass-fiber membranes. The membranes will be exposed to X-ray film. Release of TCA-precipitable $^{32}P$ from immobilized HS will indicate that the supernatant contains an endonuclease or an integrase. The supernatants from such hybridomas will be subjected to a second round of screening.

The second step will be to determine whether the $^{32}P$ from TCA-precipitable DNA is due to resolution of HS. This substrate HS will be labeled by $^{32}P$ at the 5'-ends of all four arms and incubated with these selected hybridoma supernatants. The reaction products will be resolved by electrophoresis on native 7% polyacrylamide gels before autoradiography. If the products of the reaction are one of the two pairs of DNA fragments, 60 & 53bp, or 71 & 42bp, as illustrated in Figure 5, we will conclude that the supernatant contains an integrase-like enzyme.

Hybridoma supernatants contain antibodies as well as other secretable proteins. In order to determine whether the resolution of HS is due to the antibodies in the selected supernatants, we will deplete the antibodies using Protein G Sepharose beads. If depletion of antibodies from these supernatants diminishes the resolution of HS, we will conclude that the antibody resolves HS and is a potential integrase. Its ability to catalyze DNA recombination in vitro will be examined, as described in the objective 3. The detailed methodology is given below.

*a.* Hybridoma Production: Custom immunization of mice and generation of hybridomas will be contracted to ImmunoTech Laboratories, CTTC, Houston, Texas. Mice will be immunized and anti-serum will be tested as described in Figure 6 legend. After mice show a positive ELISA, their spleens will be removed to isolate splenic cells for production of hybridomas. The fusion experiments will be performed following their routine protocol.

***Results and Discussion***: Due to US Army Medical research and Material Command Animal Use Regulations, we do not immunize mice and generate hybridomas commercially. We perform these tasks by ourselves on the campus of Baylor College of Medicine. We have successfully made 10 fusions (8 Balb/*c* mice, 1 MRL/*lpr*, and 1 NZW x BXSB) and screened over 5,000 hybridomas.

*b.* Substrates: All oligonucleotides will be synthesized and purified as described in Figure 2 legend. HS will be annealed and purified as well. Biotin will be added at the 5'-end of Oligo1 during oligomer synthesis (Genosys Biotechnologies, The Woodlands, Texas). 5'-labeling of $^{32}P$ of oligonucleotides will be carried out as described in Figure 4 legend.

***Results and Discussion***: We used two sources of oligo providers. One is Genosys Biotechnologies, The Woodlands, Texas, and the other is Gibco BRL Life Technologies. The former does not have consistent qualities for the oligos requested for purification. So we also used the latter.

*c.* Primary Screening: The primary screening substrate, HS, is formed by annealing biotinylated Oligo1 and $^{32}P$-labeled Oligo2, Oligo3 and Oligo4, as described in Figure 4 legend. The purification of this substrate is not necessary, because we measure the release of $^{32}P$ from bound substrate. Two structures, HS and Y (Figure 3), will be immobilized onto Streptavidin-coated plate because they have biotin affixed. Streptavidin will be coated on microtest plates by incubating with 5mg/ml streptavidin, 200µl/well at 4°C overnight. The plates will be washed with PBST (PBS plus 0.05% Tween-20, v/v). Biotinylated $^{32}P$-labeled HS (100µl, $5X10^4$ cpm/well) will be added for one hour at room temperature to immobilize the substrate. The wells will be washed twice with PBST to remove unbound substrate. Hybridoma supernatants (5µl) will be mixed with 95µl TSEP buffer (50mM Tris-HCl, pH7.4, 7mM spermidine, 5mM $Na_2EDTA$ and 75mM KCl, Lange-Gustafson and Nash, 1984) and incubated with immobilized substrate in the wells at 25°C for one hour. After incubation, 5µl of the reaction mixtures will be counted in a liquid scintillation counter. The remaining reaction mixtures will be mixed with ice-cold TCA to a concentration of 10% (v/v) to precipitate released DNA. The TCA-precipitated DNA will be filtered through Whatman GF/C glass-fiber membrane, and these will be exposed to X-ray film for autoradiography. The control will be the substrate incubated with the supernatant of an irrelevant hybridoma.

***Results and Discussion***: The primary screening method was not reliable due to high fluctuation of background release of bound substrate. The release was in the range of 10-30% of total bound substrate. The high fluctuation made it very difficult to interpret the data. Therefore, we skip the first step and screen every clone by electrophoresis as described in d. Determination of Catalytic Activity without removal of proteins in the samples. In order to reduce the amount of work and still obtain reliable results, we adapted a screening method described by Israel (1993). The hybridoma supernatants from 4 wells across a row or 4 wells down a column in a 96-well dish are pooled. The matrix of 16 is reduced to 8 reactions. A positive pool of an across row and a positive pool of a down column identifies a positive hybridoma clone as diagramed in Figure 8. We have not obtained a positive clone so far after screening over 5,000 hybridomas from 10 mice. Figure 9 shows one example of such screening. Although we had some candidate clones (*i.e.* Figure 9, lanes 3, 6, 8, 9, 10, and 17) that seemed to resolve HS to certain extent, we were not able to isolate single clones that possess the activity. We also observed a few hybridomas that secreted factors binding to HS in primary screenings as shown in Figure 10, lanes 4 and 5. There were 22 candidates clones went through secondary screening. Unfortunately we could not isolate these hybridomas, possessing either binding or resolving activities, after expansion and purification of the clones with extensive efforts. This failure could be due to that the initial clones were mixtures of hybridomas and the desired hybridomas grew slower than that of the other hybridomas in the same well. The growth difference resulted in loss of desired hybridomas. The other reason simply is that what we observed were artifacts.

We are interested in those hybridomas that secrete antibodies binding to HS. If we have those antibodies we can study the interaction of the antibody and the antigen, as well as engineer the antibodies by site-directed mutagenesis to reach our goals.

*d.* Determination of Catalytic Activity: Supernatants (1-2μl) which release macromolecule-bound $^{32}P$ will be incubated with gel-purified substrate HS ($5X10^3$ cpm), labeled with $^{32}P$ at the 5'-ends of all four arms, in TSEP buffer at 25°C for various time periods in a final volume of 30μl. As a control, we will use an irrelevant hybridoma supernatant. After incubation, sodium dodecyl sulfate (SDS, 10%, w/v) and proteinase K (10mg/ml) will be added to final concentrations of 0.8% SDS and 500μg/ml of proteinase K. This reaction will be carried out at 37°C for an additional 20 minutes before extraction with phenol/chloroform (1:1, v/v, phenol: TE-saturated phenol). The supernatants are then mixed with one fifth volume of loading buffer (Figure 4 legend) and resolved by electrophoresis on 7% native polyacrylamide gels (1 X TBE). The gels will be dried and exposed to X-ray film. If the supernatant contains an integrase, there should be two distinct bands in addition to the substrate HS, as illustrated in Figure 5. These bands will be purified, and their sizes confirmed, on a DNA sequencing gel (Ausubel *et al.*, 1987b). The size of each product will be compared with $^{32}P$-labeled standard molecular size markers. If the bands have the defined sizes of 60 & 53bp, or 71 & 42bp, the supernatants contain integrase-like activities.

***Results and Discussion***: Since no positive clones have been identified at this time, we have not performed this experiment.

*e.* Depletion of Antibodies from Supernatants: These selected supernatants will be incubated individually with Protein G Sepharose beads at room temperature for one hour to deplete antibodies. After incubation, the beads will be sedimented. The resulting supernatants will be tested for their ability to resolve HS as described in *c*.

***Results and Discussion***: Since no positive clones have been identified at this time, we have not performed this experiment.

Technical Objective **2**: Construction of an Fab Expression Library and Screening of Monoclonal Fabs That Resolve the Holliday Structure:

Most of the reported catalytic antibodies are monoclonal antibodies. In practice, however, isolating hybridomas that secrete antibodies with the desired catalytic activities is often a difficult and laborious job. If the efficiency of fusion of splenic cells and myeloma cells is low, one may fail to recover the cells that secrete the desired catalytic antibodies. Beyond that, the labor-intensive screening process may take many months. Recent advances in molecular biology permits the construction of Fab expression libraries, which provide an alternative approach to isolation of mono-specific antibodies.

Theoretically, an entire antibody repertoire can be expressed by an Fab expression library. This approach allows Fab cDNAs to be cloned and expressed in bacteria. The process is rapid and may be completed in a relatively short period of weeks to months. IgG Fab cDNAs are DNA copies from entire light-chain (κ) and one-third of the N-terminal heavy-chain (Fd). The composition of the Fab expression library may not necessarily represent the antibody repertoire that was secreted by the cell population from which the genes were obtained. Rather, the library is randomly assembled from a pool of IgG Fd and κ cDNAs. We will construct Fab expression libraries from spleen cells of HS-immunized mice. The libraries will express secreted Fabs using a bacteriophage T3 system (Stewart *et al.* 1995), and the culture supernatants from individual clones will be screened for the ability to resolve HS as described in the objective **1**. We will work on both hybridoma production and Fab expression library at the same time. This

will increase both the probability of isolating catalytic antibodies and the diversities of catalytic antibodies and/or Fabs that can be isolated. Following are the detailed methodology:

*a.* Construction of Combinatorial Repertoire: Fab expression libraries derived from the spleens of HS immunized mice will be constructed essentially following the methods described by Stewart et al. (1995). The construction is illustrated in Figure 11.

***Results and Discussion***: We initially defined conditions for RT-PCR amplification of antibody variable regions using normal mouse splenic RNA as describe in our 1998 annual report. Later we found that the conditions did not reproduce the same results once we used HS-immunized mouse splenic RNA to amplify the antibody variable regions. Therefore we spent additional efforts to define another sets of conditions for RT-PCR amplification of antibody variable regions using HS-immunized mouse splenic RNA. We have defined conditions to amplify 6 pairs out 7 light chains (κ) (Lc2, Lc3, Lc4, Lc5, Lc6, and Lc7, Figure 12, lanes 9-14, respectively), and 8 pairs out of 9 for $IgG_1$ heavy chains (Fd) (Hc1, Hc3, Hc4, Hc5, Hc6, Hc7, Hc8, and Hc9, Figure 12, lanes 1-8, respectively). Since we had difficulty amplifying Fd cDNA, we also amplified IgE Fd cDNA. We succeeded in 6 pairs (out of 9) for IgE (Hc3, Hc4, Hc6, Hc7, Hc8, and Hc9, Figure 12, lanes 17, 18, 20-23, respectively). During construction of Fab libraries, we found we had problems with reconstructed vector from pBP105 (a kind gift from S.J. Benkovic). After carefully examining the sequences of our reconstructed vector and the published information, we found that there are two *Spe*I sites about 50bp apart in pBP105 (Posner *et al.*, 1993). Replacement of Fd cDNA from pBP105 with our Fd cDNA fragments simply resulted in a wrong product by frame-shift. Now we have corrected the error and are able to make Fab libraries for testing.

*b.* Screening: The individual cloned bacterial culture medium will be screened for their activities of resolving HS as described in the objective 1. Bacterial culture supernatants usually contain detectable DNases. However, the assay buffer can eliminate DNase activity because the buffer contains high concentration of EDTA and is $Mg^{+2}$-free. Known integrases do not require $Mg^{+2}$ as a cofactor (Whang *et al.*, 1994), so presumably catalytic antibodies/Fabs with integrase activity will also not require $Mg^{+2}$.

***Results and Discussion***: We have tested the expression of Fab in the presence and absence of IPTG. IPTG is an inducer for expression of T3 RNA polymerase necessary for Fab expression. Figure 13 shows one example of Fab expression in *E.coli* BL21 with (lanes 1 and 2) and without (lanes 3 and 4) IPTG induction. Fab can be expressed in BL21 without IPTG induction (lanes 3 and 4) and expressed at higher level in comparison to induced (compare lanes 1 and 3, or 2 and 4). About 20% of expressed Fab is released into culture medium (compare lanes 1 and 2, or 3 and 4). The lower level of Fab expression in the presence of IPTG is due to the growth inhibition of IPTG on BL21. More cells in a given volume of culture result in more Fab. We have decided to screen our Fab libraries without addition of IPTG into the culture medium during the incubation.

We also tested some Fab clones from our primary Fab libraries. Figure 14 is an example. All the clones express some level of Fab (cell pellets: lanes 3, 5, 7, 9, 11, 13, 15, and 17; culture medium: lanes 4, 6, 8, 10, 12, 14, 16, and 18). The size of expressed product is about 25kD. Now we are improving the quality of our Fab libraries and trying to screen the clones in the libraries.

Technical Objective **3**: Site-Specific Recombination in vitro:

The goal of this project is to test the hypothesis that catalytic antibodies can recombine DNA fragments in vitro and to develop catalytic antibodies that can do so. The ability of selected catalytic antibodies (and Fabs) to catalyze recombination of two DNA duplexes of defined sequences will be tested in vitro. If the selected

catalytic antibodies (or Fabs) are integrases, these antibodies (or Fabs) should be able to catalyze a full circle of integrase-mediated DNA site-specific recombination.

The catalytic antibodies and/or Fabs will be purified first. The selected catalytic antibodies will be purified by protein G chromatography. Fabs will be purified by polyaspartic acid cation-exchange chromatography.

Two groups of substrates will be used to test this hypothesis. One group is the pair of 60 & 53bp (Oligo5, 60nt, Oligo6, 60nt, Oligo7, 53nt, and Oligo8, 53nt, Figure 15); the other is 71 & 42bp pair (Oligo9, 42nt, Oligo10, 42nt, Oligo11, 71nt, and Oligo12, 71nt, Figure 16). These DNAs are the products of two alternative HS resolutions. One pair of the substrates (*i.e.* 60 & 53bp) can undergo reciprocal strand exchange to form HS, which can then be resolved into the other pair (*i.e.* 71 & 42bp), or vice versa (Figure 17). (Note: Self-recombined products will not be detected by gel electrophoresis because they will be resolved into the same products.) The completion of this objective will demonstrate the feasibility of our long-term goals and provide the basis for a more ambitious future program to exploit catalytic antibodies for gene therapy.

*a.* Purification of Catalytic Antibodies: Catalytic antibodies will be purified from hybridoma supernatants by protein G Sepharose chromatography (Dean, 1992). The purity of isolated antibodies will be determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with silver or Coomassie brilliant blue. The antibody concentration will be determined by measuring $A_{280}$ (1 $OD_{280}$= 0.8mg/ml, Harlow and Lane, 1988b) or by the Bradford method in reference to BSA as described (Ausubel *et al.* 1987c).

***Results and Discussion***: Since no positive clones have been identified at this time, we have not performed this experiment.

*b.* Purification of Fabs: Catalytic Fabs will be purified from large E.coli cultures as described by Stewart et al. (1995). Briefly, culture supernatants will be collected by centrifugation and concentrated by ultrafiltration through a tangential flow apparatus. The concentrated material will be dialyzed against 20mM sodium MOPS, 0.4mM Ca acetate, pH6.3. Then the sample will be loaded onto a polyaspartic acid cation-exchange column; Fab fragments will be eluted with a linear gradient of Ca acetate from 1mM to 25mM and pH from 6.3 to 7.5, buffered with 40mM MOPS. Fab-containing fractions will be identified by SDS-PAGE, combined and concentrated by ultrafiltration. The final sample will be dialyzed against 100mM sodium HEPES, 50mM NaCl. The Fab concentration will be measured by the Bradford method.

***Results and Discussion***: Since no positive clones have been identified at this time, we have not performed this experiment.

*c.* Recombination *in vitro*: The substrate duplex DNAs (60bp and 53bp, or 71bp and 42bp) will be annealed from appropriate oligonucleotides and labeled with $^{32}P$ as described in Figure 4 legend. Recombination reactions will be performed as described in the objective 1 and analyzed by their sizes in reference to molecular size standards.

***Results and Discussion***: Since no positive clones have been identified at this time, we have not performed this experiment.

## KEY RESEARCH ACCOMPLISHMENTS

- Observed DNA Holliday structure binding factors in hybridoma culture supernatants;
- Constructed Fab expression libraries;

## REPORTABLE OUTCOMES

- Employment: one technician, 11/1/97-3/31/98
  one research associate, 5/1/98-present

## CONCLUSION

This is a pilot project to test the feasibility of generating antibodies that can recombine DNA fragments in vitro. Successful completion of these objectives will confirm the working hypothesis, and provide the basis for a more ambitious program to exploit catalytic antibodies for gene therapy. Our primary goal in this project is to create such antibodies, though we have not obtained such antibodies at this time. I feel that our resources are limited to successfully complete the project. This is a very technical and labor challenging project. Successful completion of this project will provide a new technology of DNA transfer for both biomedical research and therapeutic purposes.

## REFERENCE


Abremski, K.E. and R.H. Hoess (1992) Evidence for a Second Conserved Arginine Residue in the Integrase Family of Recombination Proteins. Protein Eng. 5:87-91;

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (1987a) Phosphatases and Kinases. in Current Protocols in Molecular Biology, edited by Wiley Interscience, pp3.10.3-3.10.5;

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (1987b) Denaturing Gel Electrophoresis for Sequencing. in Current Protocols in Molecular Biology, edited by Wiley Interscience, pp7.6.1-7.6.11;

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (1987c) Quantitation of Proteins. in Current Protocols in Molecular Biology, edited by Wiley Interscience, pp10.1.1-10.1.3;

Avalosse, B., F. Dupont and A. Burny (1995) Gene Therapy for Cancer. Curr. Opinion in Oncol. 7:94-100;

Bernard, O, S. Cory, S. Gerondakis, E. Webb and J.M. Adams (1983) Sequence of the Murine and Human Cellular myc Oncogenes and Two Modes of myc Transcription Resulting from Chromosome Translocation in B Lymphoid Tumors. The EMBO J. 2:2375-2383;

Dean, J.C. (1992) *in* Methods in Molecular Biology, Vol. 10, Immunological Protocols, edited by M. Monson, The Humana Press, Inc., pp43-63.

Duckett, D.R., A.I.H. Murchie, S. Diekmann, E. von Kitzig, B. Kemper and D.M.J. Lilley (1988) The Structure of the Holliday Junction, and Its Resolution. Cell 55:79-89;

Gates, C.A. and M.M. Cox (1988) FLP Recombinase Is an Enzyme. Proc. Natl. Acad. Sci. USA 85:4628-4632;

Harlow, E. and D. Lane (1988b) Chapter 8: Storing and Purifying Antibodies. In Antibodies: A Laboratory Manual. Cold Spring Harbor Lab. Press, Plainview, NY, p311;

Holliday, R. (1964) A Mechanism for Gene Conversion in Fungi. Genet. Res. 5:282-304;

Huse, W.D., L. Sastry, S.A. Iverson, A.S. Kang, M. Alting-Mees, D.R. Burton, S.J. Benkovic and R.A. Lerner (1989) Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda. Science 246:1275-1281;

Isreal, D.I. (1993) A PCR-Based Method for High stringency Screening of DNA Libraries. Nucleic Acids Res. 21:2627-2631;

von Kitzing, E., D.M.J. Lilley, and S. Diekmann (1990) The Stereochemistry of a Four-Way DNA Junction: A Theoretical Study. Nucleic Acid Res. 18:2671-2683;

Lange-Gustafson, B.J. and H.A. Nash (1984) Purification and Properties of Int-h, a Variant Protein Involved in Site-Specific Recombination of Bacteriophage λ. J. Biol. Chem. 259:12724-12732;

Posner, B., I. Lee, T. Itoh, J. Pyati, R. Graff, G.B. Thorton, R. La Polla and S.J. Benkovic (1993) A Revised Strategy for Cloning Antibody Gene Fragments in Bacteria. Gene 128:111-117;

Sambrook, J., E.F. Fritsch and T. Maniatis (1989) Recovery of Synthetic Oligonucleotides by Electrophoresis Through a Denaturing Polyacrylamide Gel. in Molecular Cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp11.23-11.28;

Stewart, J.D., I. Lee, B.A. Posner and S.J. Benkovic (1995) Expression of Properly Folded Catalytic Antibodies in Escherichia coli. in Methods in Enzymology. by Academic Press, Inc. Vol. 249, pp507-519;

Whang, I., J. Lee and M. Jayaram (1994) Active-Site Assembly and Mode of DNA Cleavage by FLP Recombinase During Full-Site Recombination. Mol. Cel. Biol. 14:7492-7498;

Yanez, R.J. and A.C. Porter (1998) Therapeutic Gene Targeting. Gene Therapy 5:149-159;

## APPENDICES

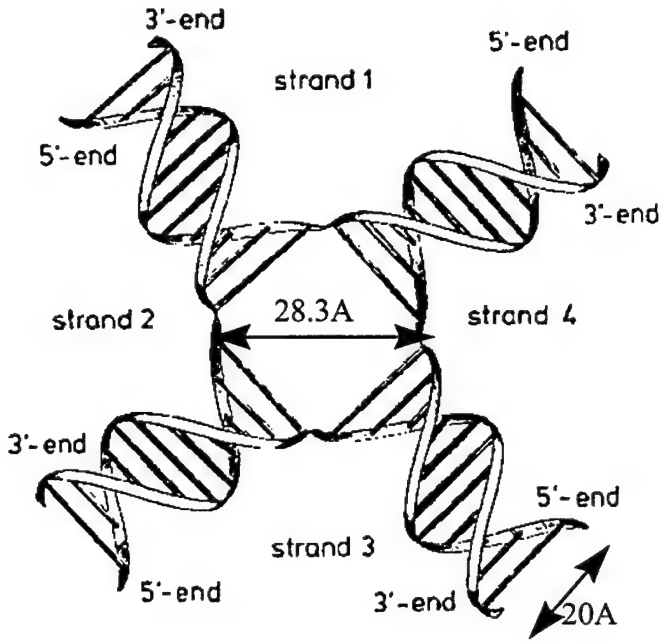


Figure 1. A ribbon model for the extended Holliday structure (von Kitzing *et al.*, 1990). The central base pairs at the junction do not stack. In each case the major groove of the base pair at the junction is pointing towards the viewer. The structure has a central size of 28.3Å. This is an X-shaped molecule that is formed from four DNA strands derived from two double-stranded DNAs. The structure is connected by a covalently closed joint (Holliday, 1964). The Holliday structure is formed by nicking and religation with the reciprocal exchange of single strands between two DNA duplexes. The structure is resolved by a second cycle of nicking and religation to form two new DNA duplexes. The formation and resolution of the Holliday structure are the processes required for integrase-catalyzed site-specific recombination.

**A.**

Oligo**1** (60nt): 5'-CGCTACAGGAGTTACAGCCTAGTAATCACACG**TCCTGC**<u>**CTCGAG**</u>**AAGGG**
**C**GTACATGCTC-3'
Oligo**2** (42nt): 5'-GAGCATGTAC**GCCCTT**<u>**CTCGAG**</u>**GCAGGA**CATCGACTAGTAGC-3'
Oligo**3** (53nt): 5'-GCTACTAGTCGATG**TCCTGC**<u>**CTCGAG**</u>**AAGGGC**CATTGAACAGTCATGCT
GTCC-3'
Oligo**4** (71nt): 5'-GGACAGCATGACTGTTCAATG**GCCCTT**<u>**CTCGAG**</u>**GCAGGA**CGTGTGATTA
CTAGGCTGTAACTCCTGTAGCG-3'

**B.**

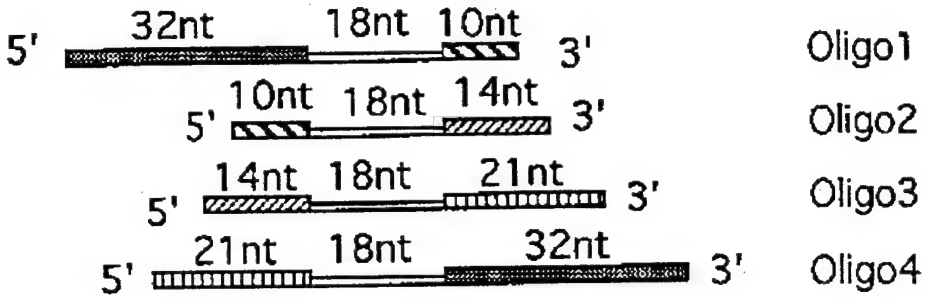


Figure 2. Oligonucleotide sequences of HS (A) and schematic diagrams of the oligonucleotides (B). All four oligonucleotide sequences have three important regions, an 18nt sequence at the center and two different lengths of flanking sequences. Bold face letters in A and open boxes in B show the 18nt central sequences. Filled boxes or cross-hatched represent the flanking sequences. The 18nt 5'-TCCTGCCTCGAGAAGGGC-3' sequences at the center of Oligo**1** and Oligo**3** are complementary to the central sequence 5'-GCCCTTCTCGAGGCAGGA-3' of Oligo**2** and Oligo**4**. This sequence has a core palindromic sequence 5'-CTCGAG-3' (an *Xho*I site, underlined) and is from human c-*myc* (-102 to -84 base pairs (bp), relative to P2 promoter; Bernard *et al.*, 1983). The flanking sequences are designed to allow HS formation. The 32nt sequence at the 5'-end of Oligo**1** is uniquely complementary to a 32nt sequence at the 3'-end of Oligo**4**. The 10nt at the 3'-end of Oligo**1** are complementary to the 10nt at the 5'-end of Oligo**2**. The 14nt at the 3'-end of Oligo**2** are complementary to the 14nt at the 5'-end of Oligo**3**. The 3'-end of Oligo**3** (21nt) is complementary to the 21nt at the 5'-end of Oligo**4**. These oligonucleotides were synthesized by solid phase automated synthesis and purified from 8% polyacrylamide-8M urea gels by standard procedures (Sambrook *et al.* 1989).

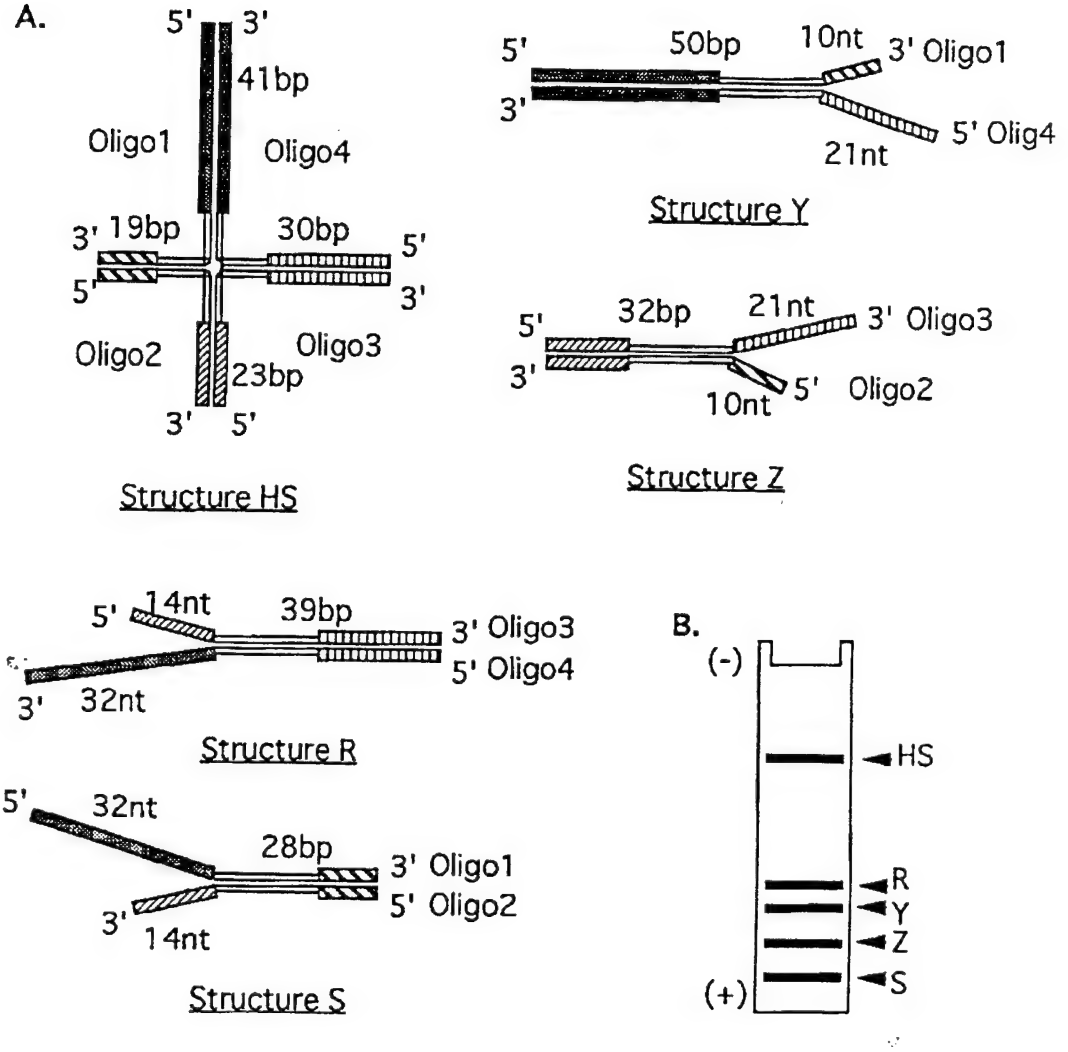


Figure 3. Schematic diagrams of predicted hybrids (A) and their relative mobilities (B). Five possible hybrids can form from a mixture of the four oligonucleotides. One will be HS. HS has four different lengths of arms (41, 30, 23 and 19bp). Other hybrids will be partial duplex DNAs annealed from the complementary sequences of Oligo**3** and Oligo**4**, Oligo**1** and Oligo**2**, Oligo**1** and Oligo**4**, and Oligo**2** and Oligo**3**. These structures are identified as R, S, Y and Z. R, S, Y and Z have different double-stranded portions of 39, 28, 50 and 32bp with two single-stranded tails, respectively. All of these hybrids have different sizes and degrees of double-stranded character, and all can be separated by their electrophoretic mobilities. HS is the largest molecule and will have the slowest mobility, as illustrated in the panel B on a native polyacrylamide gel. S is the smallest and will have the fastest mobility. Filled boxes or cross-hatched represent the flanking sequences. Open boxes represent the 18nt central sequences. The relative proportion of the hybrids differs in the hybridization mixture, because the hybrids have different $T_m$s. We routinely detect only three hybrids, HS, Y and Z, as will be shown in Figure 4.

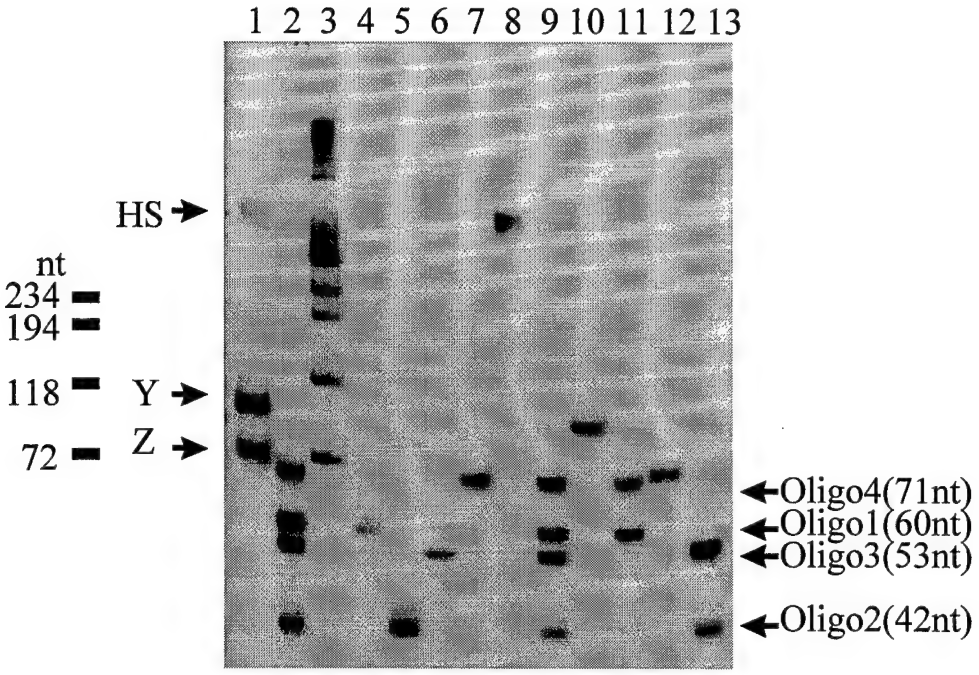


Figure 4. Formation and characterization of HS. Lane 3 is a set of $^{32}$P-labeled single-stranded molecular size markers derived by denaturing *Hae*III digested bacteriophage phiX174 DNA. Lanes 4 through 7 contain $^{32}$P-labeled oligonucleotides Oligo**1** (60nt), Oligo**2** (42nt), Oligo**3** (53nt), and Oligo**4** (71nt), respectively. The four $^{32}$P-labeled oligonucleotides have different sizes and can be distinguished by their electrophoretic mobilities on the gel. The smallest one, Oligo**2** (42nt), has the fastest mobility (lane 5); and the biggest one, Oligo**4** (71nt) has the slowest (lane 7). Stoichiometric amounts of the four oligonucleotides were mixed in TE buffer (10mM Tris-HCl, pH8.0, 1mM $Na_2EDTA$) (Duckett *et al.*, 1988), denatured at 100°C for 5 minutes, incubated at 65°C for 2 hours and allowed to cool slowly to room temperature. The mixture is resolved into three major entities, HS, Y, and Z (lane 1). This mixture of HS, Y, and Z was heat-denatured in urea to show that the mixture contained the four individual oligonucleotides (lane 2). The hybrids were purified by eluting the corresponding bands from a native 5% preparative polyacrylamide gel, with recovery by ethanol precipitation. Lanes 8, 10 and 12 are purified hybrids HS, Y and Z, respectively. Lanes 9, 11 and 13 are the denatured hybrids, respectively. Since HS has the slowest electrophoretic mobility and dissociates into a stoichiometric mixture of all four labeled component oligonucleotides, Oligo**1**, Oligo**2**, Oligo**3** and Oligo**4,** as shown in lane 9, we assumed that HS is a Holliday structure. The mixture, or the oligonucleotides or the marker were labeled by $\gamma$-$^{32}$P-ATP at their 5'-ends by T4 polynucleotide kinase according to Ausubel *et al.* (1987a). The denaturation was carried out by heating the samples in 5M urea at 95°C for 3 minutes. Non-denatured samples were mixed with one fifth volume of loading buffer (50% [v/v] glycerol, 0.025% [w/v] xylene cyanol, and 0.025% [w/v] bromphenol blue). All the samples were then loaded on a 7% native polyacrylamide gel electrophoresed in 1 X TBE buffer (100mM Tris, 2mM $Na_2EDTA$, 100mM boric acid). After electrophoresis the gel was dried and exposed to X-ray film.

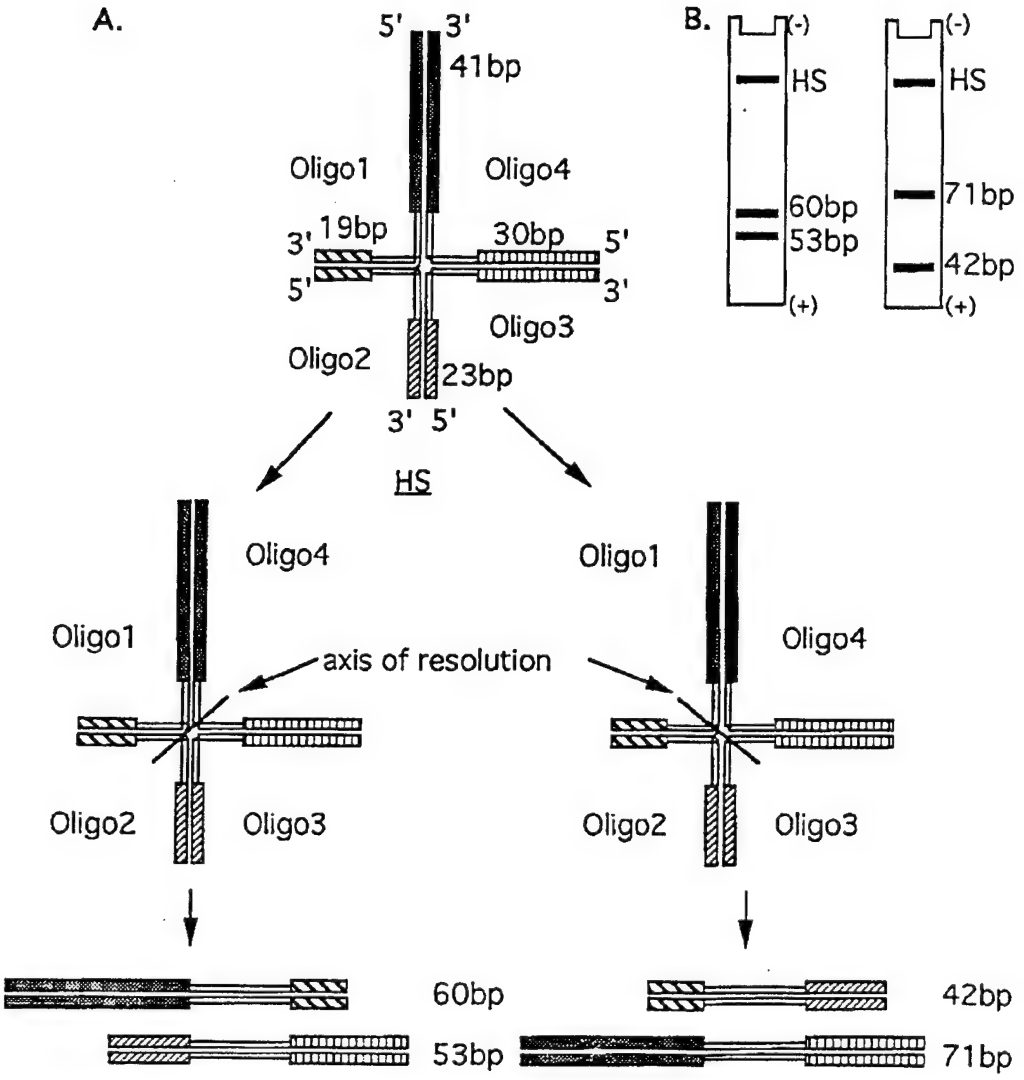


Figure 5. Schematic diagrams of HS resolution (A) and assay by gel electrophoresis (B). The resolution of HS will give rise to alternative pairs of fully double-stranded DNA products. One pair will be 60 & 53bp, the other 71 & 42bp. All these double-stranded DNA products and the hybrid HS differ in their electrophoretic mobilities under non-denaturing conditions. These mobility differences form the basis of our assay for resolution of HS. Filled or cross-hatched boxes represent the flanking sequences. Open boxes represent the 18nt central sequences (see Figure 2).

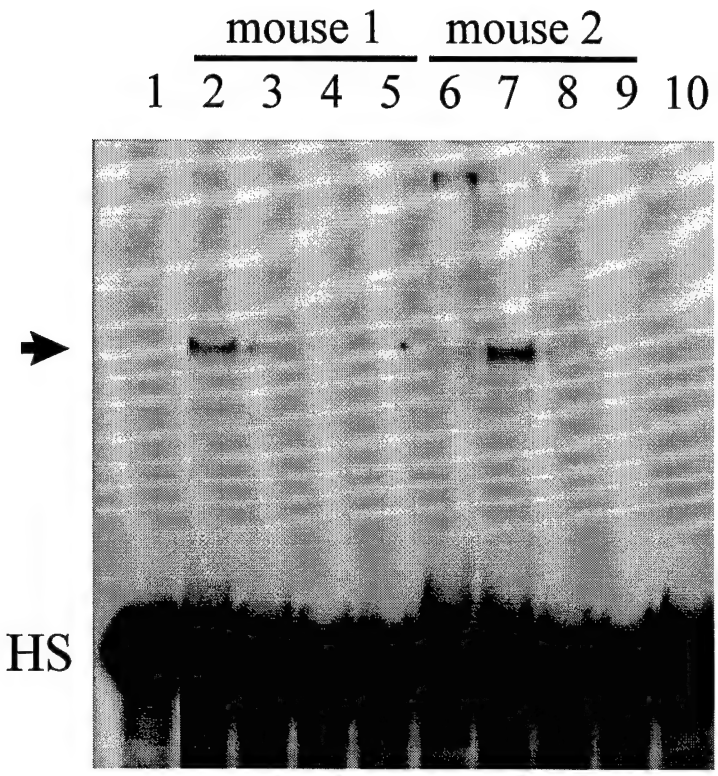


Figure 6. Formation of protein-HS complexes. The immunogen was a mixture (7.5μg) of HS, Y and Z (Figure 3) plus methylated thyroglobulin (5μg) in 10mM Tris-HCl (pH7.4), 1mM $Na_2EDTA$. Two female Balb/c mice were initially immunized with complete Freund's adjuvant, and incomplete Freund's adjuvant was used for subsequent boosts. The immunization was repeated on days 14, 28 and 42. Blood was collected on days 35 and 46, and the serum was tested for the presence of anti-HS antibodies by ELISA (Enzyme-linked Immunosorbent Assay). After the mice showed positive ELISA results, an electrophoretic mobility shift assay was performed to characterize interaction between anti-HS antibodies and purified $^{32}P$-labeled HS. All lanes contain an equal amount of $^{32}P$-labeled HS and were incubated with: lane 1, buffer alone; lane 10, normal mouse serum (1:20); lanes 2 to 5, and 6 to 9 with serial dilutions (1:20, 1:40, 1:80 and 1:160) of anti-sera from mouse 1 and 2, respectively. Sera from immunized mice contain proteins that bind stably to HS (lanes 2 and 9). Normal mouse serum does not contain HS-binding protein, as shown in lane 10. Serum or anti-serum or PBS (phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4$, 1.4mM $KH_2PO_4$, pH7.2) diluted serum (0.5μl) was incubated with purified $^{32}P$-labeled HS (2 X $10^4$ cpm, 10fmoles) in the presence of 1μg polydI.dC in 10μl. Other components in this binding system were 66.6mM KCl, 13.3mM HEPES, pH7.9, 6.6mM $MgCl_2$, 13.3% glycerol (v/v), 0.13mM $Na_2EDTA$, 0.13mM $Na_2EGTA$, pH8.0, 3mM DTT (dithiothreitol), 2μg BSA (bovine serum albumin). The binding reaction was carried out for one hour at room temperature, and the samples then were electrophoresed in a low ionic strength (0.5 X TBE) 5% polyacrylamide. The gel was dried and exposed to X-ray film. The arrow indicates the protein-HS complexes.

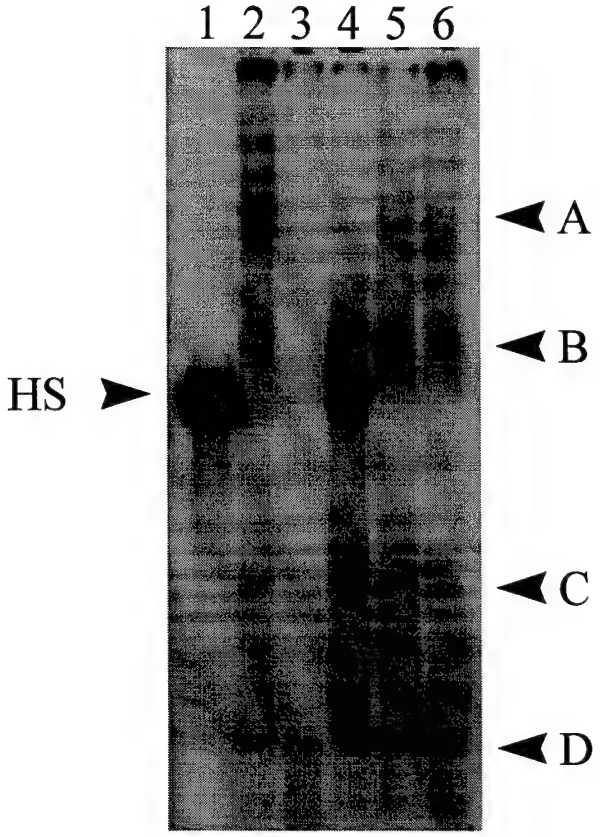


Figure 7. Binding specificity of antibodies to HS. The binding specificity of these proteins was tested, using serum from mouse 1. (**Note**: This serum was taken after one more boost to the mouse and so was different from the mouse 1 serum used in Figure 6, lane 2.). All lanes contain equal amount of purified $^{32}$P-labeled HS and were incubated with: lane 1, buffer alone; lanes 2, mouse 1 anti-serum (1:20) only; lanes 3-6, both mouse 1 serum (1:20) and goat anti-mouse IgG, purified non-labeled HS (30x in molar excess), Y (100x) and Z (100x), respectively. In the presence of the anti-serum, $^{32}$P-labeled HS was distributed into four major entities, designated A, B, C, and D (lane 2). Addition of goat anti-mouse IgG blocked the formation of A and B (lane 3). This blockage indicates that A and B are HS-antibody complexes. A could be displaced by addition of a 30-fold molar excess of non-labeled HS (lane 4). However, addition of a 100-fold molar excess of Y or Z had no effect (lane 5 and 6, respectively). The results indicate that the antibodies are saturable and bind specifically to the central structure of HS. The electrophoretic mobilities of C and D were unaltered by addition of any competitor. Since both C and D have greater electrophoretic mobilities than the probe (HS), we doubt that these are DNA-protein complexes. Finally, the electrophoretic mobilities of C and D are different from those of the dimeric hybrids that we have called Y and Z (Figure 4). Although the identity of C and D remains to be established, the electrophoretic mobility of these two entities is consistent with the hypothesis that they represent a pair of resolved, double-stranded products of an antibody-catalyzed integrase reaction. We therefore conclude that some of the antibodies in the serum may possess integrase activity. The assay condition is as described in Figure 6 legend. Double-stranded DNA was annealed by mixing stoichiometric amount of the appropriate oligonucleotides in TE buffer.

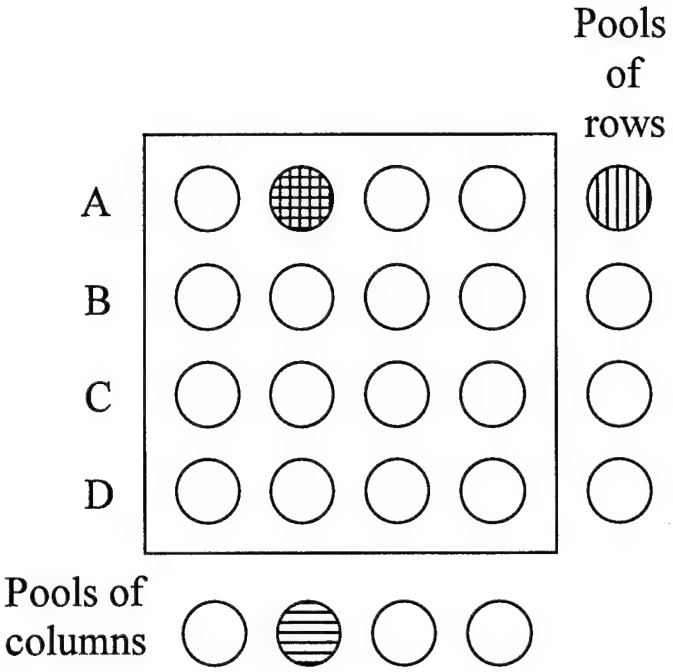


Figure 8. Schematic diagram of the screening procedure (adapted from Isreal, 1993). The hybridoma supernatants from 4 wells across a row or down a column are pooled. The matrix of 16 is reduced to 8 reactions. If both of the pools of row A and column 2 are tested positive (hatched circles). The clone at A2 position is a positive one (hatched circle).

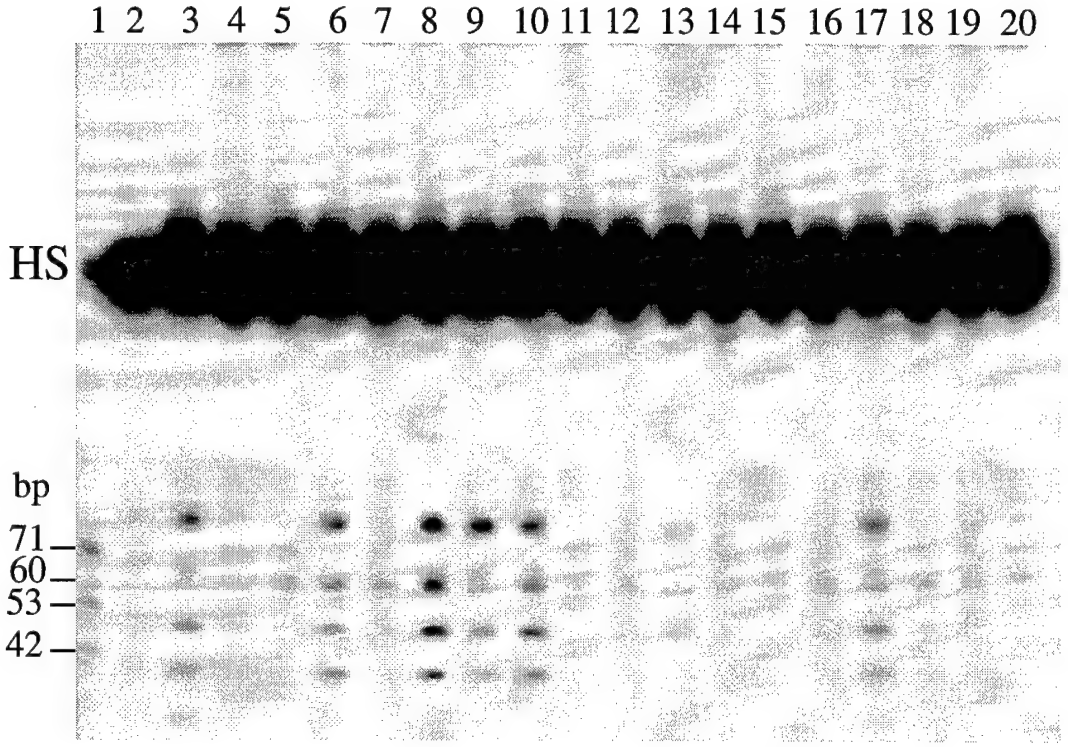


Figure 9. Examples of screening by electrophoresis (A). Lane 1 is the marker containing expected products resolved from HS. All other lanes contained equal amount of purified, $^{32}$P-labeled HS and were incubated with hybridoma culture medium (lane 2) and 18 pools of hybridoma supernatants (lanes 3-20). The reactions were carried out at room temperature for 1.5-2 hours, and the samples were then separated on 8% polyacrylamide gel by electrophoresis in 1 X TBE buffer. The gel was dried and exposed onto X-ray film.

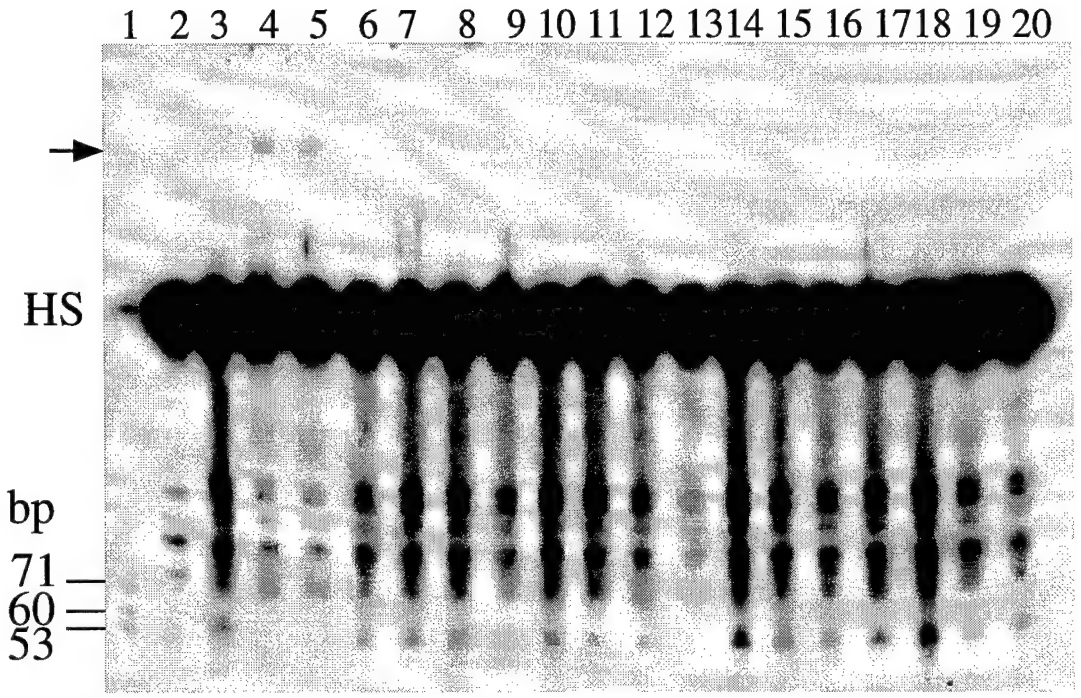


Figure 10. Examples of screening by electrophoresis (B). Lane 1 is the marker containing expected products resolved from HS. All other lanes contained equal amount of purified, $^{32}$P-labeled HS and were incubated with hybridoma culture medium (lane 2) and 18 hybridoma supernatants (lanes 3-20). The reactions were carried out at room temperature for 1.5-2 hours, and the samples were then separated on 8% polyacrylamide gel by electrophoresis in 1 X TBE buffer. The gel was dried and exposed onto X-ray film. The arrow indicates antibody-HS complex.

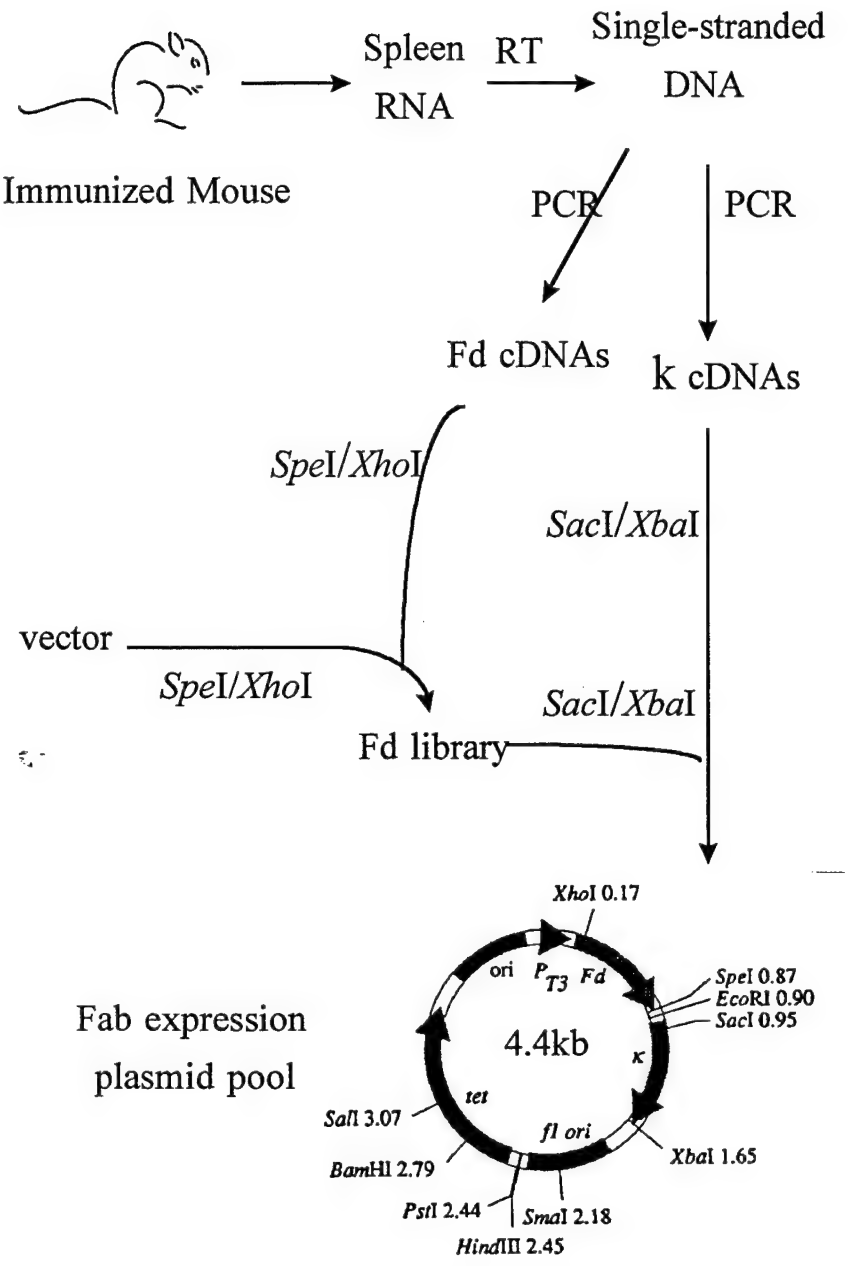


Figure 11. Construction of Fab expression library. The Fab expression library is derived from splenic RNAs. Briefly, total RNA will be isolated from spleens of HS-immunized mice. The RNAs will then be reverse transcribed into single-stranded DNA with Fd 3' and κ 3' priming. The single-stranded DNA will be used as templates for generation of IgG and IgE Fd and κ cDNA by PCR. The Fd and κ genes will be amplified separately with murine IgG primers, including one Fd 3' primer, nine Fd 5' primers, one κ 3' primer and five κ 5' primers (Huse *et al.*, 1989). Each pair of primers will give rise to a product of about 700bp. All the primers contain a restriction enzyme site: the Fd 3' primer has a *Spe*I site, the Fd 5' primer an *Xho*I site, the κ 3' primer an *Xba*I site, and the κ 5' primer a *Sac*I site. These enzyme sites allow the double-stranded PCR products to be cloned into an Fab overexpression plasmid vector. This vector features a single bacteriophage T3 promoter in front of the Fd and κ cDNAs. The PCR products will be digested by corresponding restriction enzymes and the DNA will be gel-purified. *Spe*I/*Xho*I-digested Fd cDNA will be ligated into the *Spe*I/*Xho*I sites of the vector and amplified in bacterial host. This pool of Fd-containing plasmids will then be purified, digested with *Sac*I and *Xba*I, and ligated with *Sac*I/*Xba*I-digested κ cDNA to form a second pool of Fab expression plasmids. Each plasmid in this pool will contain a randomly assembled pair of Fd and κ cDNAs, and each plasmid will express a unique Fab. For protein overexpression, the *E.coli* host will be BL21 ($F^-$, *ompT*, $hsdS_B$). Since the Fab cDNAs are under control of T3 promoter, pTG119 will be cotransformed into *E. coli* BL21 with the Fab expression plasmids. pTG119 supplies the T3 RNA polymerase under control of the inducable *lacUV5* promoter. After transformation, the *E.coli* culture will be cloned by limiting dilution. RT: reverse transcription. $P_{T3}$: T3 promoter (Stewart *et al.*, 1995).

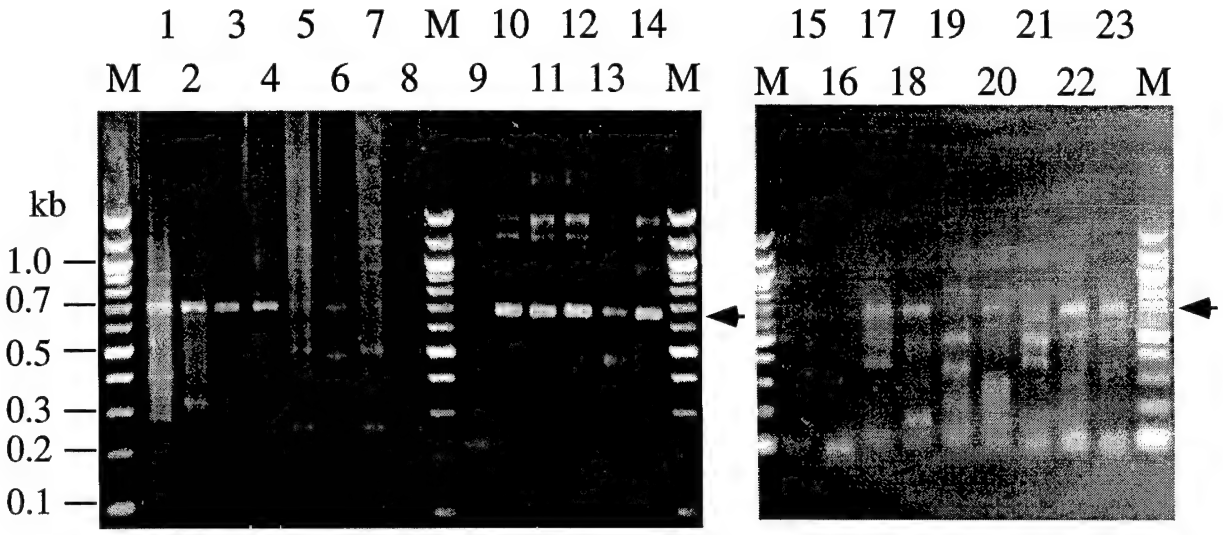


Figure 12. RT-PCR amplification of spleen Fab variable regions. Lanes M are DNA size marker (100bp DNA marker, New England BioLabs). Lanes 1-8 are $IgG_1$ heavy chains (Fd) Hc1, Hc3, Hc4, Hc5, Hc6, Hc7, Hc8, and Hc9, respectively. Lanes 9-14 are light chains (κ) Lc2, Lc3, Lc4, Lc5, Lc6, and Lc7, respectively. Lanes 15-23 are IgE Fd Hc1, Hc2, Hc3, H4, Hc5, Hc6, Hc7, Hc8, and Hc9, respectively. The arrow indicates the proper PCR products. The samples were separated on 0.8% agarose gel by electrophoresis in 1 X TBE. The gel was stained by ethidium bromide and visualized by UV emission.

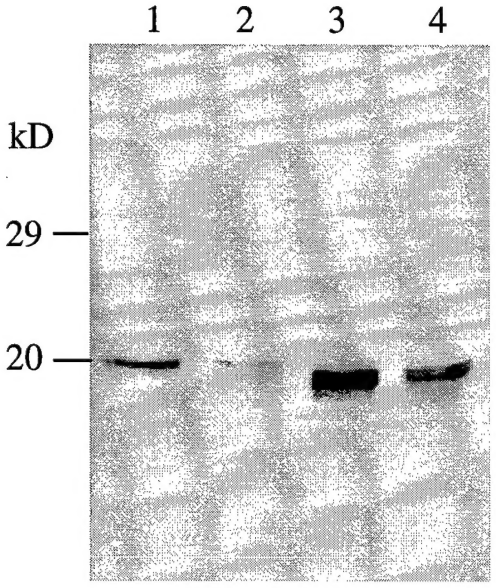


Figure 13. Expression of Fab in *E.coli* BL21 (A). Samples were prepared by growing BL21 at 37°C to reach $OD_{600}$ at 0.6. The culture was split into two. One received IPTG at a final concentration of 0.4mM, and one did not. The cells were incubated at 37°C for additional 5 hours. After incubation, cells of 20μl of each culture were pelleted. The cell pellets were lysed in 40μl of 1 X SDS sample buffer (0.05M Tris-HCl, pH6.8, 2% SDS, 10% glycerol, 50mM dithiothreitol, 0.1% bromophenol blue), and the supernatants were mixed with 20μl of 2 X SDS sample buffer. The samples were denatured at 100°C for 5 minutes, separated on 12% SDS-PAGE gel, and transferred onto nitrocellulose before immunoblotting. Lanes 1 and 2 are the cell pellet and supernatant, respectively, induced by IPTG. Lanes 3 and 4 are the cell pellet and supernatant, respectively, without IPTG induction. The arrow indicates the expressed products with apparent size about 20kD. The size marker is the pre-stained high-molecular weight marker from Gibco BRL Life Technologies.

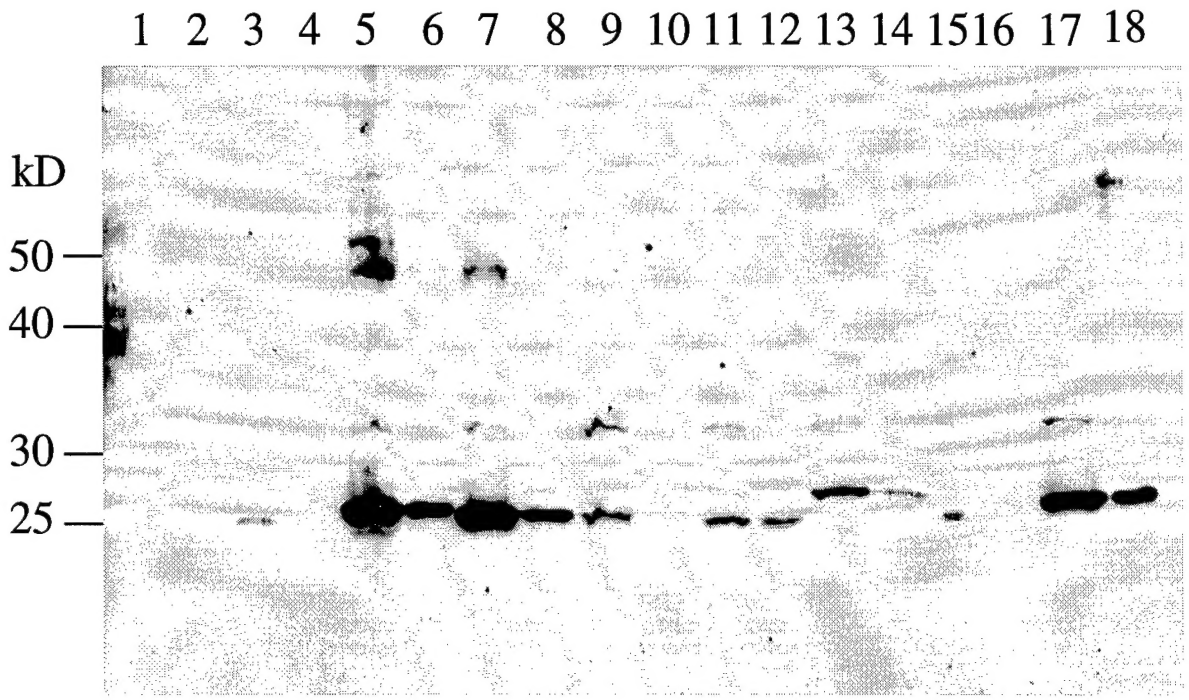


Figure 14. Expression of Fab in *E.coli* BL21 (B). Samples were prepared as described in Figure 13 legend without IPTG induction. Lanes 1 and 2 are the cell pellet and supernatant, respectively, from BL21 without expression of Fab. Lanes 3, 5, 7, 9, 11, 13, 15, and 17 are cell pellets from 8 different Fab expression clones, respectively. Lanes 4, 6, 8, 10, 12, 14, 16, and 18 are supernatants from the 8 corresponding Fab expression clones, respectively. The arrow indicates the expressed products with apparent size about 25kD. The size marker is the non-stained high-molecular weight marker from Gibco BRL Life Technologies. The size difference is due to the apparent size of molecular markers.

Oligo**5** (60nt): 5'-CGCTACAGGAGTTACAGCCTAGTAATCACACG**TCCTGCCTCGAGAAGGG**
**C**GTACATGCTC-3'
Oligo**6** (60nt): 5'-GAGCATGTAC**GCCCTTCTCGAGGCAGGA**CGTGTGATTACTAGGCTGTAA
CTCCTGTAGCG-3'
Oligo**7** (53nt): 5'-GCTACTAGTCGATG**TCCTGCCTCGAGAAGGGC**CATTGAACAGTCATGCT
GTCC-3'
Oligo**8** (53nt): 5'-GGACAGCATGACTGTTCAATG**GCCCTTCTCGAGGCAGGA**CATCGACTAG
TAGC-3'

Figure 15. Oligonucleotide sequences of duplex DNAs used in *in vitro* recombination assays (A). Bold face letters show the central sequences as indicated in Figure 2. Underlined letters are the *Xho*I sites.

Oligo**9** (42nt): 5'-GCTACTAGTCGATG**TCCTGCCTCGAGAAGGGC**GTACATGCTC -3'
Oligo**10** (42nt): 5'-GAGCATGTAC**GCCCTTCTCGAGGCAGGA**CATCGACTAGTAGC-3'
Oligo**11** (71nt): 5'-CGCTACAGGAGTTACAGCCTAGTAATCACACG**TCCTGCCTCGAGAAGG**
**GC**CATTGAACAGTCATGCTGTCC-3'
Oligo**12** (71nt): 5'-GGACAGCATGACTGTTCAATG**GCCCTTCTCGAGGCAGGA**CGTGTGATTA
CTAGGCTGTAACTCCTGTAGCG-3'

Figure 16. Oligonucleotide sequences of duplex DNAs used in *in vitro* recombination assays (B). Bold face letters show the central sequences as indicated in Figure 2. Underlined letters are the *Xho*I sites.

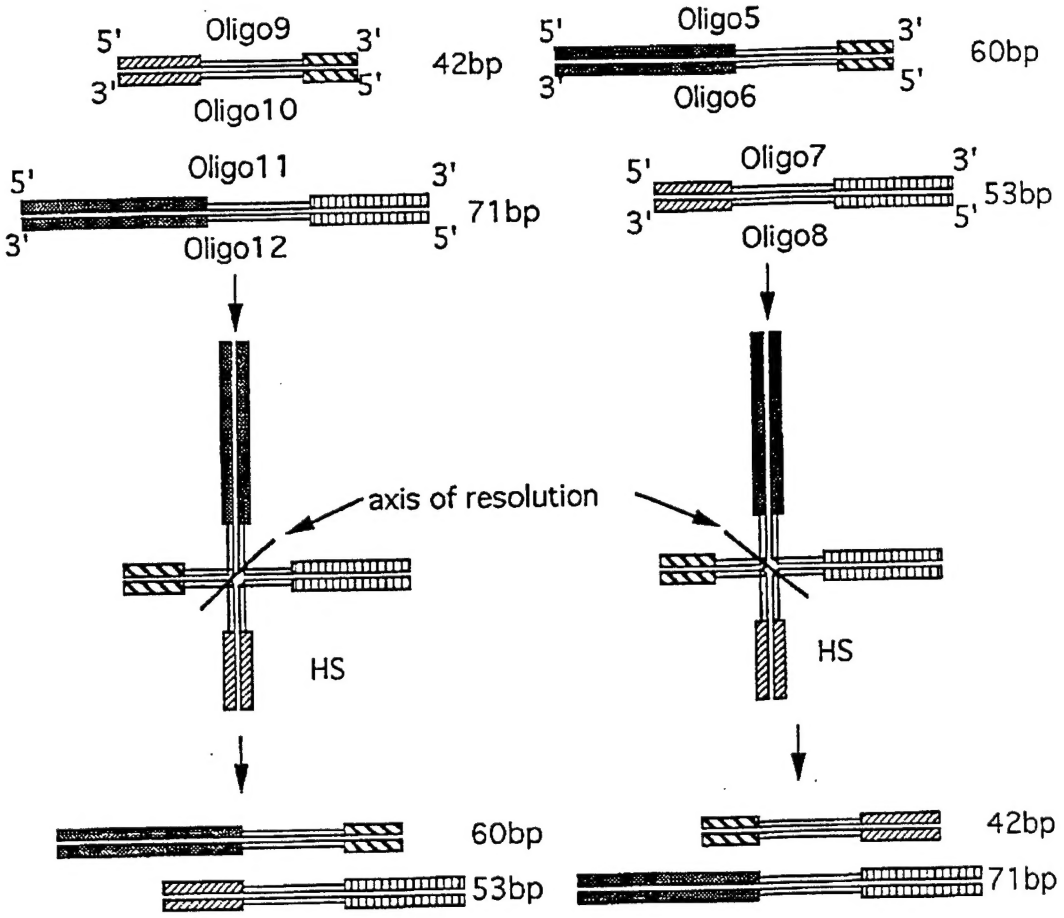


Figure 17. Schematic diagram of DNA recombination *in vitro*.